pH-dependent insertion of proteins into membranes: B-chain mutation of diphtheria toxin that inhibits membrane translocation, Glu-349 → Lys

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ABSTRACT To investigate how diphtheria toxin (DT) undergoes pH-dependent membrane translocation in mammalian cells, we have isolated and characterized mutants of the toxin that are defective in acidic-pH-dependent killing of *Escherichia coli*. Cloned DT secreted to the periplasm of *E. coli* kills the bacteria under acidic conditions (near pH 5.0) by inserting into and permeabilizing the inner membrane (a mechanism independent of the toxin's ADP-ribosylation activity). Mutant forms of DT with reduced lethality for *E. coli* were selected by plating the bacteria under acidic conditions. CRM503, one of the full-length mutants selected by this protocol, also showed diminished cytotoxicity for mammalian cells. We traced the altered cytotoxicity of CRM503 to a Glu-349 → Lys mutation (E349K), one of three point mutations, within the B fragment. The E349K mutation alone inhibited cytoxicity and membrane translocation in mammalian cells and lethality for *E. coli* but did not affect enzymic activity or receptor binding. The recently determined crystallographic model of DT shows that Glu-349 resides within a short loop connecting two long hydrophobic α-helices of the translocation domain. Protonation of Glu-349 and two other nearby acidic residues, Asp-352 and Glu-362, may enable these helices to undergo membrane insertion and the intervening loop to be transferred to the opposite face of the bilayer. The E349K mutation introduces a positive charge at this site, which would be expected to inhibit membrane insertion and the insertion-dependent activities of DT. These results suggest that protonation of Glu-349 and nearby acidic residues may be important in triggering the translocation step of toxin action.

Bacterial proteinaceous toxins that insert into and traverse membranes offer interesting opportunities to study the interactions of proteins with lipid bilayers. Diphtheria toxin (DT, 535 residues) belongs to a class of toxins that enzymically modify substrates within mammalian cells (1). Many of these toxins are initially taken into cells by receptor-mediated endocytosis, and subsequently, an enzymically active polypeptide moiety of the toxin is released into the cytosol from endosomes or another membrane-bound compartment. For DT, a proteolytic fragment (fragment A, or DTA) is transferred to the cytosol and catalyzes the ADP-ribosylation of elongation factor 2 and thereby inhibits protein synthesis and causes death of the cell (2).

How the enzymically active moiety crosses a membrane is not well-understood for any toxin but, for DT and certain other toxins (and for many animal viruses, as well), acidic intravesicular pH is known to trigger the process. A variety of evidence indicates that the membrane translocation event for DT occurs when the toxin is exposed to a pH near 5.0 in

the endosomal compartment (3–6). *In vitro*, treatment of DT with buffer of pH near 5.0 induces a conformational change that causes the toxin to insert into artificial lipid bilayers (7–10), as manifested, for example, by the formation of ion-conductive membrane channels (11–14). Such channels have been observed both in natural and artificial membranes, but their relationship to the translocation of DTA remains uncertain (15, 16).

The B chain of DT (DTB) mediates membrane insertion, channel formation, and translocation of DTA, and hydrophobic regions present within DTB have been proposed to function in these activities (17, 18). Although the effects of pH on the conformation have been studied by various methods, there is little information available about the precise mechanism of triggering by acidic conditions (8, 19). The current mutant search was undertaken in hopes of identifying one or more specific amino acid residues within DTB that are involved in triggering the acidic-pH-dependent conformational change and translocation.

Although many mutant forms of DT have been isolated (20, 21), heretofore, it has not been possible to use positive genetic selection to search for such mutants, because no selective advantage or disadvantage to toxin production *in vitro* was known. Here we have used a positive selection protocol based on our original report of acid-dependent sensitivity of *Escherichia coli* that are synthesizing cloned DT (22). [In compliance with the National Institutes of Health recombinant DNA guidelines for cloning DT in *E. coli*, we used an enzymically attenuated form of the toxin, DTS, that contains an active-site mutation, E148S (22, 23).] The toxin is secreted to the periplasm of *E. coli*, and when the bacteria are treated with acidic buffer, the protein apparently inserts into and permeabilizes the inner membrane. Thus the lethal action of DT for *E. coli* depends on its ability to permeabilize membranes and is unrelated to its ADP-ribosyltransferase activity. Mutant forms of DTS selected for their inability to kill *E. coli* might, therefore, be expected to include ones that are defective in membrane interactions.

Here we report the isolation and characterization of three full-length DT mutants by this approach. One of them, CRM503, proved to be markedly less toxic for mammalian cells than the parental DTS. The defect of CRM503 in cytotoxicity and in pH-dependent membrane translocation was traced to E349K, one of three point mutations within the B fragment. The location of the E349K mutation in the

recently determined crystallographic model of DT suggests an explanation for its effects and an important role for Glu-349 in the acidic-pH-triggered translocation event (24).

EXPERIMENTAL PROCEDURES

Bacterial Strains and Vectors. *E. coli* JM103 was the host for all plasmids used. pDO1 is a pBR322-derived plasmid containing DNA sequences encoding DTs under control of the inducible *tac* promoter. For mutagenesis, two M13 vectors were constructed. (*i*) The *Bam*HI–*Hin*dIII segment of ptacF2 (22), which encodes the entire F2 fragment (25) except for eight C-terminal amino acids, was cloned into the identical sites in M13mp19 (M13mp19::F2 *Bam*HI–*Hin*dIII). (*ii*) Similarly, the *Cla* I–*Sph* I segment of pDO1, encoding 57% of fragment B, was cloned into the compatible *Acc* I–*Sph* I sites of M13mp19 (M13mp19::DT *Cla* I–*Sph* I). To utilize the *Pvu* II site in the DTB region of pDO1 for subcloning, an 873-base-pair *Ava* I–*Nde* I fragment carrying a *Pvu* II site in the vector region was deleted from pDO1, creating pDO1PvuII.

Mutagenesis and Selection Protocol. Nitrous acid mutagenesis was performed for 1 h as described (26, 27) on DT gene fragments cloned into M13 (M13mp19::F2 *Bam*HI–*Hin*dIII or M13mp19::DT *Cla* I–*Sph* I). After synthesis of double-stranded DNA, mutagenized gene fragments encoding portions of DTB were removed from the M13mp19 vectors and cloned into unmutagenized pDO1. The *Nsi* I–*Cla* I fragment (267 base pairs) from M13mp19::F2 *Bam*HI–*Hin*dIII and the *Pvu* II–*Sph* I fragment (573 base pairs) from M13mp19::DT *Cla* I–*Sph* I were cloned into the corresponding sites in pDO1, and the ligation products were used to transform *E. coli* JM103 (28). The transformed cells were allowed to grow for 1 h in L broth at 37°C and were plated on L agar plates of acidic pH, containing 50 mM sodium citrate, 50 mM sodium succinate, and ampicillin (100 μg/ml). Survivors were chosen for further analysis.

CRM503 was isolated as follows. After growth for 1 h, transformed cells were diluted into L broth containing ampicillin (100 μg/ml) and allowed to grow overnight at 37°C on a shaker. The next day the cultures were streaked on L agar plates at pH 5.0. Individual colonies were picked, grown in L broth, and screened for DT-related proteins by Western blot analysis. CRM501 and CRM502 were isolated from transformed cells plated directly at pH 5.5.

DTs-E349K, DTs-N399D, and DTs-V443I were constructed by directed mutagenesis in M13mp19::DT *Cla* I–*Sph* I and subsequent subcloning of the *Pvu* II–*Sph* I fragment carrying each mutation, into the corresponding sites of pDO1PvuII. The base alterations were confirmed by DNA sequencing, according to Sanger *et al.* (29), with the Sequenase system obtained from United States Biochemical.

Production of DT-Related Proteins. Overnight cultures were diluted in L broth or M9 medium containing ampicillin (100 μg/ml) and grown at 37°C to OD595 1.2–2.0. The cultures were then induced by addition of isopropyl β-D-thiogalactoside to 1 mM and harvested 1–3 h later by centrifugation. Periplasmic extracts were prepared as described (30, 31). DT-related proteins were initially isolated from these periplasmic extracts by three consecutive steps of column chromatography (FPLC, Pharmacia) on Mono Q HR 5/5, phenyl-Superose, and Superose 12 HR 10/30 columns. In later preparations, periplasmic extracts were fractionated with ammonium sulfate prior to chromatography on a Mono Q HR 5/5 column.

Protein Synthesis Assays. BS-C-1 cells (African green monkey kidney cells, ATCC CCL 26) and Vero cells (African green monkey kidney cells, ATCC CCL 81) were used interchangeably as toxin-sensitive mammalian cell lines. The cells were routinely grown in an atmosphere of 7.5% CO₂/92.5% air in Eagle's minimal essential medium supplemented with nonessential amino acids and Earle's salts containing 5% (vol/vol) fetal bovine serum, penicillin G (100 units/ml), and streptomycin (100 μg/ml). To measure the cytotoxicity, confluent monolayers of cells in 24-well assay plates were incubated at 37°C in growth medium containing 5% of the normal amount of L-leucine. During the last hour of exposure to the toxins, the cells were incubated in the presence of L-[3,4,5-³H]leucine (1 μCi/ml; 1 Ci = 37 GBq) to measure protein synthesis by incorporation of radioactivity into acid-insoluble material (6).

Toxin Competition Assays. Competition of unlabeled toxin mutants with ¹²⁵I-labeled wild-type DT (specific activity, 10⁷ cpm/μg) for binding to toxin-sensitive cells was performed essentially as described (32), in the same growth medium used in the cytotoxicity assay. Specific binding was determined by subtracting radiolabel bound by cells incubated with ¹²⁵I-labeled DT plus a 200-fold excess of unlabeled toxin from the label bound in the presence of ¹²⁵I-labeled DT alone. Nonspecific binding of ¹²⁵I-labeled DT was 20–25% of the total binding.

Nicking of DT-Related Proteins. DTs or mutant toxins in 50 mM Tris·HCl, pH 7.6/1 mM CaCl₂ were nicked in the presence of 1 mM NAD and L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (1 μg/ml) for 15 min at 25°C. The reaction was stopped by the addition of soybean trypsin inhibitor to 5 μg/ml. SDS/polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol showed that each protein was completely nicked.

Other Methods. SDS/polyacrylamide gel electrophoresis was by the method of Laemmli (33). Western blot analysis was performed as described (25) except that rabbit anti-DT antibody (Connaught Laboratories) was detected by goat anti-rabbit antibodies conjugated to alkaline phosphatase (ICN). ADP-ribosylation of elongation factor 2 *in vitro* was carried out as described (34) except that the reaction was done at 37°C and the specific activity of [*adenylate*-³²P]NAD (New England Nuclear) was adjusted to 5 Ci/mmol.

RESULTS

Nitrous acid was used to mutagenize single-stranded DNA fragments encoding portions of the B fragment of DT cloned within bacteriophage M13. After conversion of the M13 DNA to the double-stranded form, restriction fragments specifying portions of DTB were excised and exchanged into the unmutagenized pDO1 vector, which encodes the full-length DTs. The ligated vector was then used to transform *E. coli* JM103, and the cells were plated at acidic pH. Clones of pDO1 containing mutagenized DNA fragments survived growth on acidic agar plates (pH 5.0 or 5.5) at a frequency of 1–3%, under the conditions employed. Survivors were picked and grown overnight in L broth containing ampicillin (100 μg/ml). Aliquots of these cultures were then lysed and examined for toxin-related protein by Western blot analysis. Clones producing full-length forms of DTs constituted ≈5% of the survivors. The other survivors contained DT-related polypeptides with molecular masses <45 kDa, which probably resulted from nonsense mutations, missense mutations that sensitized the protein to proteolytic attack, or cloning artifacts.

Three full-length mutant forms of DTs—CRM501, CRM502, and CRM503—were chosen for characterization. All three were found to have multiple single-base mutations within DTB, as determined by DNA sequencing. Two substitutions were found in CRM501—G268E (GGG → GAG) and V283A (GTA → GCA); two were found in CRM502—M230I (ATG → ATA) and E232K (GAA → AAA); and three were found in CRM503—E349K (GAG → AAG), N399D (AAC → GAC), and V443I (GTT → ATT).
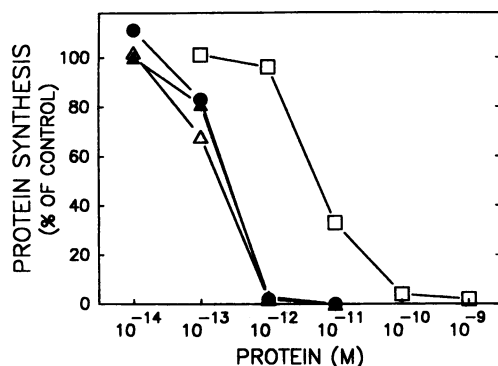
FIG. 1. Inhibition of protein synthesis in BS-C-1 cells by DT_S, CRM501, CRM502, and CRM503. Cells were incubated in the presence of various concentrations of the nicked toxins for 24 h and assayed for capacity to synthesize protein during the last hour of incubation. ●, DT_S; △, CRM501; ▲, CRM502; □, CRM503.

The CRM501, CRM502, and CRM503 proteins were purified from periplasmic extracts of *E. coli* JM103 and analyzed. On SDS/polyacrylamide gels each preparation showed a single major band that reacted with anti-DT antibodies in Western blots (data not shown). DT_S and the three derivative proteins were all predominantly in the unnicked form and were nicked by mild trypsin treatment before cytotoxic activity was measured.

Relative cytotoxic activities of DT_S and the three mutants were quantified by assaying inhibition of protein synthesis in toxin-sensitive mammalian cells. In a 24-h assay, CRM503 exhibited ≈20-fold lower specific cytotoxicity than DT_S (Fig. 1), whereas CRM501 and CRM502 showed no change relative to the parental toxin. Control experiments showed that the presence of contaminating periplasmic proteins, even at levels much higher than those in our purified toxin preparations, did not affect the cytotoxicity assay (data not shown).

To test CRM503 for a possible defect in membrane translocation, we measured its ability to undergo acid-induced translocation across the plasma membrane (6, 35). Cells were incubated with CRM503 or DT_S for 2 h in the presence of NH_4Cl, a lysosomotropic agent that blocks intoxication by raising the pH within normally acidic intracellular compartments. The cells were then treated for 10 min at various pH values, incubated for 24 h, and assayed for protein synthetic
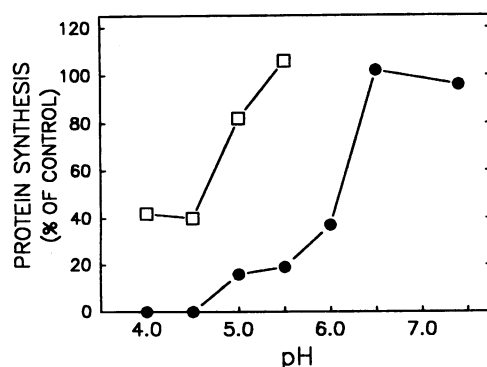

FIG. 2. pH-mediated entry of DT_S and CRM503 into BS-C-1 cells. Cells were incubated at 37°C with 20 mM NH_4Cl throughout the experiment. After a 15-min preincubation in fresh growth medium, the cells were exposed to 1 nM nicked toxins for 2 h. The medium was exchanged for growth medium lacking NaHCO_3 containing 25 mM Hepes at various pH values, and 10 min later this medium was exchanged for growth medium containing 5% of the normal concentration of L-leucine. Protein synthesis was measured after 24 h and is expressed as a percentage of controls incubated in the absence of toxin. ●, DT_S; □, CRM503.
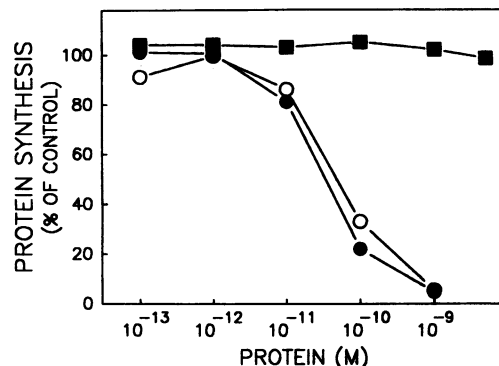

FIG. 3. Inhibition of protein synthesis in Vero cells by DT_S, DT_S-E349K, and DT_S-V443I. Cells were incubated for 5 h at 37°C in the presence of the nicked proteins. Protein synthesis is expressed as a percentage of radioactivity incorporated into cells in the absence of toxin. ●, DT_S; ■, DT_S-E349K; ○, DT_S-V443I.

activity. As shown in Fig. 2, to achieve 50% inhibition of protein synthesis, CRM503 required a pH ≈1.5 units lower than that required with DT_S. The ability of nicked CRM503 to compete with ^{125}I-labeled DT for binding to receptors on BS-C-1 cells was unaltered relative to control DT_S. ADP-ribosyltransferase activity, a property of free DTA, was also unaffected, as expected (data not shown).

We employed directed mutagenesis to introduce the three point mutations of CRM503 individually into DT_S, to determine which mutation was responsible for its functional defect. DT_S-E349K and DT_S-V443I were produced by *E. coli* at levels similar to the parental toxin. The N399D mutation proved destabilizing, however, and DT_S-N399D could never be obtained, even from *E. coli* strains deficient in the production of cytoplasmic [Y1083 (36)] or periplasmic proteases [KS474 (37)]. Therefore, we partially purified DT_S-E349K and DT_S-V443I from *E. coli* periplasmic extracts and characterized them. Both proteins showed one major band on Western blots, with the expected molecular mass.

DT_S-E349K was similar to CRM503 in all properties tested, whereas DT_S-V443I was essentially identical to DT_S. DT_S-E349K showed no cytotoxicity at 5 nM, the highest concentration tested (Fig. 3), and none was seen when cell-surface-bound DT_S-E349K was treated with pH pulses as low as pH 4.4 (Fig. 4). Lower pH values caused loss of cell viability even in the absence of toxin. Neither receptor-binding activity nor ADP-ribosyltransferase activity was affected by the
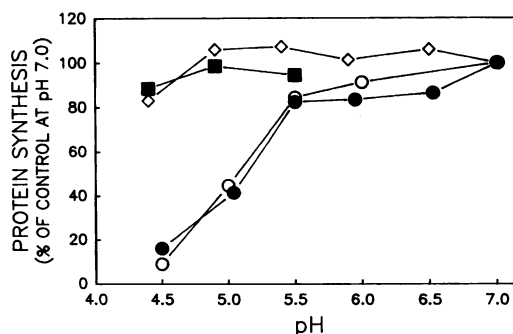

FIG. 4. pH-mediated entry of DT-related proteins into Vero cells. Cells were preincubated 20 min at 37°C in bicarbonate-free growth medium containing 25 mM Hepes and 10 mM NH_4Cl and then incubated 1 h with nicked proteins at 5 nM. After washing, the cells were pulsed with the above medium at various pH values for 15 min. Protein synthesis was measured 20 h after the pH pulse. Protein synthesis is expressed as a percentage of radioactivity incorporated into cells when pulsed at neutral pH. ◇, Vero cells in absence of toxin; ●, DT_S; ■, DT_S-E349K; ○, DT_S-V443I.

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E349K mutation (data not shown). *E. coli* synthesizing DTₛ-E349K exhibited resistance to plating at low pH similar to that seen in bacteria producing CRM503 (Fig. 5). DTₛ-V443I was identical to control DTₛ with regard to cytotoxicity, pH-mediated entry, and lethality for *E. coli* (Figs. 3–5).

DISCUSSION

The process of membrane translocation of DT in mammalian cells, the killing of *E. coli* synthesizing DTₛ, and the formation of channels by DT in native or artificial membranes involve similar regions of polypeptide within DTB and are triggered by acidic conditions near pH 5.0. This suggests that common structural elements and similar conformations of the B chain may mediate these various phenomena and, thus, that one should be able to obtain individual mutations within DTB affecting all of them. Consequently, some of the mutations that impair the toxin's lethality for *E. coli* should also affect its membrane interactions and cytotoxicity in mammalian cells. Here we have selected and defined one such mutation, E349K.

One of three full-length mutant toxins selected for impaired lethality for *E. coli*, CRM503, proved to have reduced cytotoxic activity for toxin-sensitive mammalian cells as well. This molecular phenotype was found to correlate with the presence of E349K, one of three point mutations within CRM503. The E349K mutation alone impairs pH-dependent translocation across the plasma membrane of toxin-sensitive mammalian cells, and also, presumably, across endosomal membranes, thereby inhibiting cytotoxicity. The fact that receptor-binding and ADP-ribosylation activities were unaffected by E349K implies that the defect in toxin action on mammalian cells is limited to the translocation step. Both DTₛ-E349K and CRM503 also show a major reduction in channel-forming activity in planar lipid bilayers at low pH (V.C., D.O.O., J. Mindell, A. Finkelstein, and R.J.C., unpublished results). The E349K mutation thus causes a general impairment of the low-pH-dependent interaction of DT with bilayers.

The location of Glu-349 within the recently determined crystallographic model of DT (24) is consistent with the proposal that protonation of this residue may play a crucial role in membrane insertion. Glu-349 resides within the α-helical T (transmembrane) folding domain, which is distinct from the C (catalytic) and R (receptor-binding) domains. Glu-349 and another acidic residue, Asp-352, constitute the only ionizable residues within a short loop (TL5) connecting two long apolar α-helices (TH8 and TH9). This helix–loop–helix (Fig. 6) constellation may represent an insertion "dagger" that penetrates into an adjacent bilayer when the two carboxylate groups are protonated. The apolar helices may
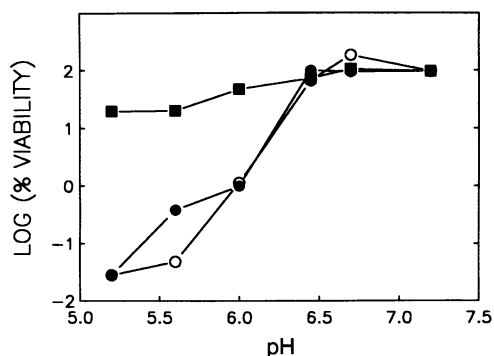

FIG. 5. Viability of *E. coli* harboring plasmids encoding DT-related proteins when plated on medium of various pH values. Viability was compared to controls plated at neutral pH. ●, DTₛ; ■, DTₛ-E349K; ○, DTₛ-V443I.
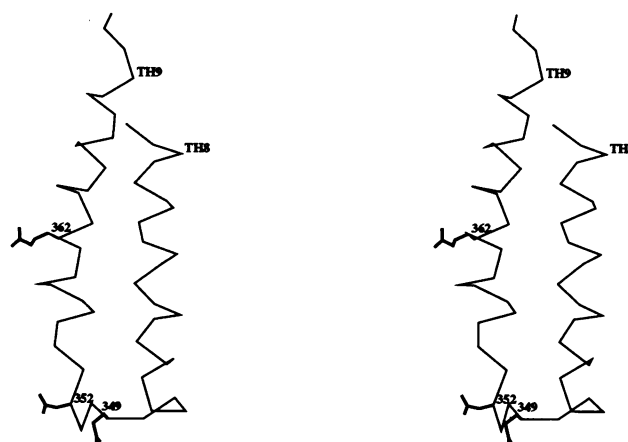

FIG. 6. Stereoscopic diagram of apolar helices TH8 and TH9 of DT with connecting loop TL5. A trace of the α-carbon backbone is shown and the side chains of Glu-349, Asp-352, and Glu-362 are indicated. The structure shown is derived from the work of Choe *et al.* (24).

then span the bilayer, with the TL5 tip exposed to the aqueous phase on the opposite face of the membrane. The E349K mutation, by introducing a positively charged group, would be expected to impede insertion of the TL5 loop and inhibit insertion-dependent functions, including cytotoxicity.

An additional acidic residue, Glu-362, which is centrally located within the TH9 helix, would be buried within the apolar phase of the membrane according to this model, and presumably this residue also would need to be protonated for insertion to occur. In other studies, we have shown that mutation of an isoleucine within TH9 (Ile-364) to lysine also blocks cytotoxicity and channel formation of DTₛ (V. Cabiaux *et al.* unpublished results).

If the pH on the trans face of the membrane were approximately neutral, as expected for the cytosol, the side-chain carboxyl groups of Glu-349 and Asp-352 should become deprotonated and thereby anchor the toxin in the membrane. This mechanism may account for the observation that channel formation depends on the difference in pH across the membrane (12). Whether or not other structural elements of the B chain also undergo insertion is difficult to predict, but a second set of hydrophobic helices within DTB, TH5–TH7, forms another interesting candidate for an insertion dagger. Loop TL3, between helices TH5 and TH6, also contains acidic residues and may represent an insertion tip for this constellation. Helices TH1–TH4, which comprise the remainder of the T domain, are very hydrophilic and unlikely to undergo membrane insertion.

How the insertion of helices TH8 and TH9, and possibly of TH5–TH7, serves to facilitate transfer of DTA across a membrane is open to speculation. It is known from studies with membrane-restricted photoprobes that fragment A can enter the bilayer at acidic pH, and it may be that the primary role of the translocation domain is to tether fragment A closely to the membrane and thereby increase the probability of its insertion and passage across the bilayer. Further studies will be required to distinguish between this model and others in which fragment B plays a more complex or specific role. For example, the unfolded A chain has been proposed to pass through an aqueous channel formed by the B chain (12).

Glu-349 represents, to our knowledge, the first amino acid residue to have been identified in DT, or any other toxin, that is likely to function directly in regulating the pH-dependent triggering of membrane insertion. Two other point mutations in the B chain have been reported to cause a defect specifically in the translocation function of DT, but the functional alterations caused by these mutations are likely to be due to

nonspecific conformational alterations. Johnson and Youle (38) found that the P308S substitution, which lies within the TH5–TH7 constellation, decreased efficiency of translocation 10-fold, but only when the toxin was bound by a surrogate receptor. No difference relative to the control was found when the toxin was bound by the DT receptor. Mutation of Cys-471 to Tyr (39), which disrupts a disulfide bridge within the R domain, has been reported to cause a 5000-fold loss of cytoxicity due to a defect in the translocation function. How the specific C471Y mutation within R affects the translocation function remains to be determined. This loss of function is not due merely to disruption of the disulfide bridge, however, since replacement of both Cys-461 and -471 with Ser did not affect translocation (40).

The pH-dependent selection described here for mutations affecting lethality of DT for *E. coli* represents, to our knowledge, the first positive selection protocol to have been applied to the isolation of mutant toxins. There is not a perfect correlation between loss of lethality for *E. coli* and loss of cytotoxic activity for mammalian cells, for unknown reasons, but the yield of mutants with the latter phenotype is at least reasonably good, based on the limited sample characterized. Neither CRM501 nor CRM502 proved to be defective in pH-dependent channel formation, which is consistent with the fact that neither was altered in cytotoxic activity. Positive selection approaches similar to that described here may be applicable to isolation of mutant forms of other pH-dependent toxins or other proteins known to undergo condition-dependent membrane interaction.

The results presented here support a model for the pH-dependent insertion of segments of a water-soluble protein into a lipid bilayer. It will be interesting to see if the helix–acidic tip–helix motif is observed in other toxins and animal viruses that use low pH as a trigger for traversing membranes (11, 41–43).

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